Mechanisms of tumor resistance to 4-hydroperoxycyclophosphamide (4-HC) were studied by using a panel of human medulloblastoma cell lines either passed in the laboratory for resistance to 4-HC or established from tumors showing clinical resistance to cyclophosphamide. The use of an inhibitor of 4-HC metabolism, 2-methylmercaptopropionamide, allowed the comparison of 4-HC-resistant lines with closely related parental control lines. Several of the cell lines [D283 Med (4-HCR), D341 Med (4-HCR), D458 Med] contained elevated levels of glutathione (GSH). No changes in glutathione-5-transferase activity or isozyme pattern were observed, but in two of these three lines, the elevation in GSH was accompanied by elevated levels of γ-glutamyl transpeptidase. To confirm the role of elevated GSH content in 4-HC resistance, the sensitivity of the cell lines to 4-HC was repeated after depletion of GSH by treatment with l-buthionine-S,S-sulfoximine. No change was observed in Daoy (4-HCR) and D458 Med cell lines abolished when 4-HC was replaced with phenylketocyclophosphamide, a 4-HC analogue that cannot be detoxified by ALDH. Thus, elevated levels of ALDH appear to play a role in the resistance of Daoy (4-HCR). Several of the cell lines [D283 Med (4-HCR), D341 Med (4-HCR), Daoy (4-HCR), D458 Med] contained elevated levels of glutathione (GSH). No changes in glutathione-5-transferase activity or isozyme pattern were observed, but in two of these three lines, the elevation in GSH was accompanied by elevated levels of γ-glutamyl transpeptidase. To confirm the role of elevated GSH content in 4-HC resistance, the sensitivity of the cell lines to 4-HC was repeated after depletion of GSH by treatment with l-buthionine-S,S-sulfoximine. In medulloblastoma cell lines without other mechanisms of resistance, a linear relationship was seen between GSH content and resistance to 4-HC. Moreover, cells with GSH content >5 nmol/mg protein and no other overriding mechanism of resistance could be sensitized to 4-HC treatment with l-buthionine-S,S-sulfoximine. Finally, D283 Med (4-HCR) cells had mild elevations in both ALDH and GSH content, but were resistant to phenylketocyclophosphamide and were not significantly sensitized by l-buthionine-S,S-sulfoximine. This cell line appears to demonstrate a third mechanism of resistance to 4-HC. These results suggest that 4-HC resistance in medulloblastoma can be multifactorial.

INTRODUCTION

Cyclophosphamide is one of the most active chemotherapeutic agents identified to date for the treatment of medulloblastoma. Radiographic evidence of cyclophosphamide-induced tumor regression has been seen in patients with recurrent (1, 2) and newly diagnosed medulloblastoma (3). Studies in progress are seeking to extend these Phase II data by demonstrating the efficacy of cyclophosphamide in prolonging survival of patients with newly diagnosed high-risk medulloblastoma. Unfortunately, emergence of drug-resistant tumor cells invariably leads to tumor progression and subsequent death, warranting efforts to define the mechanisms responsible for cyclophosphamide resistance and modulations effective in bypassing or reversing this resistance (4).
produced by centrifugation (105,000 x g for 60 min at 4°C) of homogenates prepared in 0.1 M potassium phosphate buffer (pH 7.4) and treated with three cycles of freezing and thawing. The assay was performed immediately after extraction with a reaction mixture containing 100 μM NAD, 1 mM propionaldehyde, 0.1 M potassium phosphate buffer (pH 7.4), and 100 μg of protein/ml. NADH formation was measured by using a Gilford Fluoro-4 spectrofluorometer (Gilford Institute Laboratories, Oberlin, OH) at excitation 350 nm, emission 460 nm.

Measurement of γ-GTP. γ-GTP activity was measured by the method of Tate and Meister (20). Cultured cells were washed twice with Dulbecco’s phosphate-buffered saline and suspended in 0.01 M Tris- HCl (pH 8.0) containing 0.15 M NaCl, giving a final cell count of 10^6/ml or protein concentration of 1 to 2 mg/ml. The assay solution contained 0.1 ml of cell suspension, 2.5 mM L-γ-glutamyl-p-nitroanilide, 30 mM glycylglycine (NaOH added to give pH 8.0), and 50 mM Tris-HCl (pH 8.0) in a final volume of 1 ml. The solutions were incubated at 37°C in a shaking water bath for 30 min. Vials with intact cells were chilled to 0°C to stop the reaction and were immediately centrifuged. Vials with cell homogenates were put in boiling water for 1 min and then centrifuged. In either case, the amount of p-nitroaniline in the supernatant was determined by using a Gilford Response spectrophotometer (Gilford Institute Laboratories) at 410 nm (ε = 8800 M^-1 cm^-1). Activity was expressed as nmol of p-nitroaniline formed per mg of protein per min.

Protein Measurement. Protein was measured by the method of Bradford (21).

Drugs. 4-HC was provided by Nova Pharmaceutical (Baltimore, MD). BSO was synthesized as previously described (22). PKCP was synthesized as previously described (23).

Limiting Dilation Analysis. Cells were harvested in exponential growth and treated at a density of 10^6 cells/ml with 4-HC or PKCP (concentrations, 250, 100, 50, 10, 5, 0 μM) for 1 h. 4-HC or PKCP was then washed out, serial 5-fold dilutions were made from each original tube, and 100 μl were plated per well in a 96-well flat-bottomed tissue culture plate (6 wells/dilution, 8 dilutions/original tube). The plates were incubated at 37°C in a humidified atmosphere of 5% CO2 for 12 days. Each well was scored as positive (21 colony) or negative for the presence of colonies. The number of colonies per treatment group versus control (drug vehicle alone) was analyzed using an inverted microscope. Each well was scored as positive (≥1 colony) or negative for the presence of colonies. The number of colonies per treatment group versus control (drug vehicle alone) was analyzed by Spearman analysis to estimate the log kill in each treatment group.

BSO Treatment. Cells treated with BSO were exposed to a concentration of 100 μM for 24 h prior to measurement of GSH content or treatment with 4-HC for limiting dilution analysis as described above.

Statistical Analysis. Statistical significance was assessed by the Wilcoxon rank sum test.

RESULTS

Derivation of Cell Lines. A description of the cell lines characterized in these studies is detailed in Table 1. Two cell lines, D341 Med and Daoy, were derived from primary cerebellar tumors at the time of initial diagnosis of a medulloblastoma. D283 Med was derived from the malignant ascites and peritoneal implants of a recurrent medulloblastoma following failure of radiotherapy. D283 Med (4-HCR), D341 Med (4-HCR), and Daoy (4-HCR) were generated by serial pulse treatment with increasing doses of 4-HC. D384 Med was derived from a newly diagnosed medulloblastoma metastatic to the subarachnoid space in a 17-month-old boy who subsequently failed therapy with cyclophosphamide. D425 Med was derived from the primary cerebellar tumor in a 6-year-old boy; D458 Med was derived later from tumor cells in the cerebrospinal fluid of the same patient following failure of radiotherapy plus chemotherapy (cyclophosphamide, vincristine, cisplatin).

Designation of 4-HC Resistance. Three cell lines, D283 Med (4-HCR), D341 Med (4-HCR), and Daoy (4-HCR), are resistant to 4-HC as compared with their respective parental lines (Table 2) and are considered to have in vitro-generated resistance. D384 Med, derived from cerebellar primary medulloblastoma and shown to be 4-HC resistant, is considered to have de novo clinical resistance. D458 Med, isolated following failure of therapy in a patient whose primary tumor was used to establish D425 Med, is considered to have acquired clinical resistance.

GSH, γ-GTP, and GST. The GSH content and activities of γ-GTP and GST are detailed in Table 3. There was a significant (P < 0.05) increase in the GSH content of D283 Med (4-HCR), D341 Med (4-HCR), and Daoy (4-HCR) cells compared with their respective parent lines. γ-GTP was significantly (P < 0.05) elevated in two of these three lines, Daoy (4-HCR) and D341 Med (4-HCR).

No significant increase in GST activity was seen in any resistant cell line (Table 3). Measurements of total GST activity, however, do not address the hypothetical possibility that a single isoform of GST might be expressed at higher levels in the 4-HC-resistant lines and that the other isoforms of GST might be coordinately down-regulated. To examine this possibility, Western blotting was performed by using antisera raised against the various classes of human GST (Fig. 1). GST-π was the predominant isoform detected in the medulloblastoma cell lines (Fig. 1B). Within each pair of samples, the level of GST-π was the same in the resistant cell line and in its sensitive counterpart. Low levels of GST-π were detected in D384 Med, the cell line with the lowest levels of GST-π (Fig. 1C), but not in any of the other cell lines. GST-μ was not detected in any of the cell lines (Fig. 1D) and remained undetectable even upon prolonged exposure of the blot. There was no evidence for the enhanced expression of a particular isoform of GST in the resistant cell lines compared with their sensitive counterparts.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell line derivation</th>
<th>Growth pattern</th>
<th>Karyotype</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>D283 Med</td>
<td>Malignant ascites and peritoneal implants from 6-year-old boy with recurrent medulloblastoma following failure of radiotherapy</td>
<td>Suspension</td>
<td>47,XY,+11,i(17q),8q*,20q*</td>
<td>10</td>
</tr>
<tr>
<td>D283 Med (4-HCR)</td>
<td>Serial pulse treatment of D283 Med with increasing doses of 4-HC</td>
<td>Suspension</td>
<td>47,XY,+6,+8,+18,+22, +1p–(+17q),+DMs</td>
<td>11</td>
</tr>
<tr>
<td>D341 Med</td>
<td>Biopsy of cerebellar primary tumor in 4-year-old boy</td>
<td>Adherent</td>
<td>Tetrapol</td>
<td>9</td>
</tr>
<tr>
<td>Daoy (4-HCR)</td>
<td>Biopsy of cerebellar primary tumor in 4-year-old boy</td>
<td>Suspension</td>
<td>Tetraploid</td>
<td>9</td>
</tr>
<tr>
<td>D384 Med</td>
<td>Biopsy of cerebellar primary tumor in 17-mo-old boy who subsequently failed initial therapy with cyclophosphamide</td>
<td>Adherent</td>
<td>46,XY,–5,8(17q)+,8q+, +10q–</td>
<td>12</td>
</tr>
<tr>
<td>D425 Med</td>
<td>Tumor cells in cerebrospinal fluid following failure of radiotherapy plus chemotherapy (cyclophosphamide, cisplatin, vincristine) in same patient from which D425 Med was derived</td>
<td>Suspension</td>
<td>46,XY,i(17q),10q–,+DMs</td>
<td>12</td>
</tr>
<tr>
<td>D458 Med</td>
<td>Tumor cells in cerebrospinal fluid following failure of radiotherapy plus chemotherapy (cyclophosphamide, cisplatin, vincristine) in same patient from which D425 Med was derived</td>
<td>Suspension</td>
<td>47,XY,+18,17q,6q+, +10q–,+DMs</td>
<td>12</td>
</tr>
</tbody>
</table>
Modulation of GSH Content with 4-HC Sensitivity by BSO. Following incubation with BSO for 24 h, the GST content of the medulloblastoma cell lines was reduced. Cellular contents (nmol/g protein) were as follows: D283 Med, 2.3 ± 0.6; D283 Med (4-HCR), 1.9 ± 1.3; D341 Med, 4.1 ± 0.9; D341 Med (4-HCR), 3.0 ± 1.0; Daoy, 1.1 ± 0.3; Daoy (4-HCR), 0.9 ± 0.1; D384 Med, 2.0 ± 0.6; D425 Med, 2.2 ± 1.2; and D458 Med, 2.0 ± 0.8. In general, residual GSH in the parental and resistant lines was approximately 17.5 and 10.0% of controls, respectively.

Preincubation of the medulloblastoma cell lines with BSO produced heterogeneous effects on 4-HC cytotoxicity (Table 4). BSO significantly enhanced 4-HC cytotoxicity against D341 Med and D341 Med (4-HCR) with complete restoration of 4-HC sensitivity in D341 Med (4-HCR) to parental levels. Similar results were seen with D425 Med and D458 Med, with complete restoration of parental sensitivity. In contrast, BSO treatment of Daoy and Daoy (4-HCR) produced some enhancement of 4-HC cytotoxicity, particularly in Daoy (4-HCR), but without restoration of sensitivity to parental levels. Finally, BSO did not sensitize D283 Med or D283 Med (4-HCR) to 4-HC.

ALDH Levels and PKCP Sensitivity. Measurements of ALDH (Table 3) revealed that the enzyme was undetectable in most of the cell lines. There was a significant (P < 0.05) but small increase in ALDH activity in the Daoy (4-HCR) line compared with that of the parent line, and an even smaller increase in the D283 Med (4-HCR) line compared with D283 Med.

To assess whether these increases in ALDH activity might be playing a role in 4-HC resistance, the cytotoxicity of PKCP (a cyclophosphamide analogue that is not metabolized by ALDH) was assessed. The doses of PKCP that produced a 1-log kill of Daoy and Daoy (4-HCR) were 12.9 ± 2.6 and 22.4 ± 6.5 μM (P < 0.01), respectively. Thus, the Daoy (4-HCR) cells, which were 6-fold more resistant to 4-HC than the parent (Table 2), were less than 2-fold resistant to PKCP. This observation suggests that the elevated ALDH level observed in the Daoy (4-HCR) cell line might contribute to 4-HC resistance. In contrast, the doses of PKCP that produced a 1-log kill of the D283 Med and D283 Med (4-HCR) were 13.1 ± 3.7 and 65.9 ± 28.3 μM (P < 0.01), respectively. Thus, D283 Med (4-HCR) is cross-resistant to 4-HC and PKCP.
was observed to be 8-fold more resistant to 4-HC but only of ALDH were demonstrated in the Daoy (4-HCR) compared observed in these cells (32). In the present study, elevated levels of ALDH activity contributes to the resistance of Daoy (4-HCR) substrate for ALDH. These observations suggest that elevated levels of ALDH resistance of neoplastic cells to alkylating agents such as 2-fold more resistant to PKCP, a 4-HC analogue that is not a with the parental Daoy line (Table 3). Moreover, Daoy(4-HCR) resistance appears to result from the high levels of ALDH. 

DISCUSSION

Treatment of medulloblastoma remains a difficult challenge. The majority of patients with this tumor die of progressive disease following failure of surgical and radiotherapeutic intervention (4). The demonstration that cyclophosphamide, cisplatin, and vincristine are active against medulloblastoma (26) generated hope that adjuvant chemotherapy might substantially increase the survival of patients with this tumor. Clinical experience, however, suggests that the majority of patients treated with these agents also develop progressive tumor (27-29). Thus, identification of the mechanisms of drug resistance and development of strategies for bypassing this resistance are important research goals in this disease.

Previous studies have demonstrated the cytotoxicity of 4-HC against human medulloblastoma cell lines in culture and of cyclophosphamide against human xenografts growing in athymic nude mice (30). We subsequently established that elevated 4-HC-resistant sublines by serial pulse exposure of parental lines to increasing doses of 4-HC. Additional 4-HC-resistant cell lines were established from primary tumors demonstrating de novo or acquired resistance to cyclophosphamide in the clinical setting. In the present paper, the mechanisms of resistance to 4-HC in these cell lines have been characterized. The results suggest that resistance to 4-HC in medulloblastoma cell lines involves at least three different mechanisms: elevated levels of ALDH, elevated levels of GSH, and a third, currently uncharacterized mechanism.

Elevated levels of ALDH facilitate the detoxification of cyclophosphamide by catalyzing conversion of aldosphamide to arboxyphosphamide (31). Resistance of L1210 murine leukemia cells to cyclophosphamide based on this mechanism has been reported by Hilton (5). Furthermore, the relative resistance of hematopoietic stem cells to cyclophosphamide-induced cytoxicity appears to result from the high levels of ALDH observed in these cells (32). In the present study, elevated levels of ALDH were demonstrated in the Daoy (4-HCR) compared with the parental Daoy line (Table 3). Moreover, Daoy(4-HCR) was observed to be 8-fold more resistant to 4-HC but only 2-fold more resistant to PKCP, a 4-HC analogue that is not a substrate for ALDH. These observations suggest that elevated ALDH activity contributes to the resistance of Daoy (4-HCR) to 4-HC.

The remainder of the resistance of the Daoy (4-HCR) line appears to be related to the elevated levels of GSH. GSH is a ubiquitous tripeptide that plays a central role in the protection of cells against a spectrum of toxic insults (33). Previous studies have demonstrated an association between elevated GSH levels and resistance of neoplastic cells to alkylating agents such as melphalan (8) and cyclophosphamide (6). More recently, GSH conjugates of melphalan (34), nitrogen mustard (35), chlorambucil (36), and cyclophosphamide (37) have been demonstrated by high-pressure liquid chromatography, raising the suggestion that GSH can detoxify all of these alkylating agents and prevent cross-link formation. Consistent with this suggestion, depletion of GSH levels by treatment with BSO has resulted in increased sensitivity to alkylating agents in vitro (38-41) and in vivo (42, 43). In the present study, the Daoy (4-HCR) cell line was shown to contain 2-fold more GSH than the parental cell line. Moreover, resistance of the Daoy (4-HCR) cell line to 4-HC was diminished approximately 2-fold in the presence of BSO (Table 3). These results prompt us to suggest that the resistance of the Daoy (4-HCR) cell line to 4-HC results in part from the elevated expression of ALDH (see above) and in part from the elevated level of GSH.

Elevated levels of GSH also appear to play a role in the resistance of several of the other medulloblastoma cell lines to 4-HC. D341 Med (4-HCR) is modestly resistant (1- to 4-fold) to 4-HC compared with D341 Med. Not only were elevated levels of GSH observed in the D341 Med (4-HCR) line compared with the D341 Med line (Table 3), but BSO had a larger effect on the sensitivity of the D341 Med (4-HCR) line that it did on the D341 Med line (Table 4). Likewise, the D341 Med line, isolated from a patient who did not respond to cyclophosphamide treatment, had a somewhat elevated content of GSH and was sensitized to 4-HC by BSO. Finally, the resistant lines D458 Med and D425 Med contained high levels of GSH. The sensitization of these cell lines by BSO is also consistent with a role for elevated levels of GSH in the 4-HC resistance phenotype.

In Fig. 2, the relationship between 4-HC sensitivity and cellular GSH concentration is shown for all of the cell lines except Daoy (4-HCR), a cell line in which elevated ALDH contributes to resistance (see above), and D283 Med (4-HCR), a cell line exhibiting a third mechanism of resistance (see below). In the cell lines that do not have other clear-cut mechanisms of resistance, there appears to be a linear relationship between GSH content and 4-HC sensitivity. This relationship holds in the absence and presence of BSO treatment to deplete GSH levels. The data points for D283 Med (4-HCR) and Daoy (4-HCR) lie very far above the regression line, even when these cell lines are pretreated with BSO. The regression lines for the remaining cell lines are shown because they manifest additional mechanisms of resistance (see text).

\[ y = 6.67 + 0.324x \]
\[ R^2 = 0.647 \]
also noteworthy to emphasize that all of the cell lines except D283 Med (4-HCR) were sensitized to 4-HC by incubation with BSO, indicating that cellular GSH content greater than ~5 nmol/mg protein produces resistance to 4-HC.

An additional mechanism of resistance to alkylating agents in other cell types appears to involve changes in GST rather than elevations of GSH per se. Elevated levels of the GST have been demonstrated in a diverse spectrum of alkylator-resistant cell lines to 4-HC. Moreover, an altered distribution of isoenzymes without changes in chlorodinitrobenzene-measured activity has been observed in certain resistant cells (7). In the present study, there was no evidence for an increase in GST activity (Table 3) or a change in distribution of the various isoenzymes (Fig. 1, B–D) in the 4-HC-resistant medulloblastoma lines. GST does not appear to play a role in resistance of medulloblastoma cell lines to 4-HC.

The preceding observations appear to account for 4-HC resistance in all of the cell lines except D283 Med (4-HCR). D283 Med (4-HCR) is 8-fold resistant to 4-HC compared with the parental cell line (Table 2). This cell line is 5-fold resistant to PKCP, an analogue that is not affected by ALDH. Moreover, this cell line remains fully resistant after depletion of GSH with BSO (Table 3). These observations suggest that the major mechanism of resistance in this cell line does not involve either elevated ALDH or GSH. O’Connor et al. (44) have recently described a cell line that appears to have enhanced resistance to 4-HC and B-D) in the 4-HC-resistant medulloblastoma lines. GST does not appear to play a role in resistance of medulloblastoma cell lines to 4-HC.

In summary, we have demonstrated that many 4-HC-resistant medulloblastoma cell lines to 4-HC.

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